Shoot tissue culture of *Robinia pseudoacacia* f. *decaisneana*

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Abstract: *Robinia pseudoacacia* f. *decaisneana* is a transfiguration of *Robinia pseudoacacia*. For enhancing propagation coefficient of the species, the experiment of shoot tissue culture of *Robinia pseudoacacia f. decaisneana* was conducted in Forestry College of Shenyang Agricultural University from July 1999 to July 2001. The experiment included medium selection of explant induction survival, initial culture, subculture as well as rooting culture, and forming seedling with callus. The results showed that shoot segment *in vitro* survive rate is larger in spring than in autumn, and green dense callus could form plantlet. The best medium for initial culture was SH+0.5mg/L BA+0.05 mg/L NAA, with a propagation coefficient of 4.1 (per micro-cutting in a month), and for subculture it was B_5+0.5 mg/L BA+0.05 mg/L NAA+ 10 mg/L Glu., with a propagation coefficient of 4.7. The best rooting medium was 1/2MS+0.5 mg/L NAA+10 mg/L Glu., with a rooting rate of 84.4%. These results provide reference data for reproduction of superior individuals of *Robinia pseudoacacia f. decaisneana*.

Keywords: Robinia pseudoacacia f. decaisneana, Shoot segment, Tissue culture, Medium

Abbreviations: BA: Benzyladenine; NAA: Naphthalene acetic acid; 2,4-D: 2,4-dichlorophenoxyacetic acid; NAA :naphthalene acetic acid; LH: Lactalbumin hydrolysate; GA gibberellic acid; Vc: vitamin C.

CLC number: S792.270.5; S722.3 **Document code:** A **Article ID**: 1007-662X(2002)01-0051-05

Introduction

Robinia pseudoacacia is a multipurpose species (Bi 1995). Traditional reproductive method cannot meet the needs of production because of low propagation rate and long propagation periods. Since the end of 1950s', the tissue culture of *Robinia pseudoacacia* has been conducted and culminated after 1980s'. Nowadays some achievements have been made in culture of cambium, shoot segment, leaves, embryo, isolation and culture of protoplast as well as gene engineering (Wang 1999). At the same time, some studies have been done in forming seedling with callus.

Propagation from root cuttings and tissue culture are valuable for reproduction of superior individuals or varieties of black locust (Redei 2001). The most researches on tissue culture focused on shoot segment culture of black locust, and a number of good varieties and clones have been obtained. For example, Jaszkiseri propagated greatly in New Zealand (Barghchi 1987, Chalupa 1987). Three clones in Shandong Province, China (Wang 1988) and 9 clones in Hebei Province (Ji 1993) were selected and reproduced. Using shoot segment for tissue culture is the most efficient propagation method and the most case-hardened method in explant tissue culture. The shoot segments collected from locust juvenile tree (Chalupa 1989), or the same-year branch (Davis 1987), have greater regeneration ability, with characteristics of quicker cell dedifferentiation, more propagated buds, easily rooting for young stems, and lower dosage of growth hormone needed in every culture period (Kamlesh 1995). $AgNO_3$ is benefit to the in vitro culture of shoot segment. It can reduce the exosmosis of tannin and the release of ethane and stop the forming of callus of stem basis, thus benefiting the propagation of stem (Barghchi 1987; Jong 1995).

Cambium cells have the capability of differentiation and regeneration ability of plantlet. We can use cambium tissue from explants of shoot tip or waster shoot of black locust to culture and form callus, and then differentiate buds (Han1993; Han 1997; Degchi 1994). The cambium from waster shoot could produce more buds than that from shoot tip. Yucel (1988) reported that root phellem cambium tissue could form seedling in vitro.

Callus can form plantlet effectively. Jong (1998) reported that green-yellow and bright and dense callus from shoot base could be differentiated easily, formed plantlet on MS basic medium with various concentration BA and NAA, and rooted on 1/2MS medium, thus a forming plantlet propagation system by callus could be established successfully.

Tissue culture of black locust has been studied widely and thoroughly (Wang 1999), but many varieties and clones have not been studied systematically so far. *Robinia pseudoacacia* f. *decaisneana* is a transformation of *Robinia pseudoacacia*, belonging to defoliate shrub. It has creeper shoot, odd number feather leaves, raceme axillary growth, amaranth anadem butterfly forming. In China the early planting of this species is in Beijing, and now it is also cultivated in Dalian, Xiongyue, Xingcheng, Shenyang of

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Received date: 2001-12-30
Responsible editor: Chai Ruihai

Liaoning Province (Li 1990, 1999). However *Robinia pseudoacacia* f. *decaisneana* in vitro culture has not been reported. The authors of this paper studied the tissue culture of *R. pseudoacacia* f. *decaisneana* shoot segment and determined the optimal medium and season of explant induction, optimal sterilizing time, and the transplanted times on the same initial medium. Meanwhile, the optimal subculture and root medium was selected and forming plantlet by callus was explored.

Material and method

Explants induction in spring and autumn

The browses of *R. pseudoacacia f. decaisneana* were collected in March and cultured in water with the addition of 10 mg/L GA_3 and 100 mg/kg Vc. Two weeks later, the branches sprouted and they were washed with washing powder, then in running water (tap water) for 10 min. Shoot segments with 1~2 axillary buds were firstly sterilized 30 s in alcohol, then sterilized for 3 min (no petiole) and 5 min (with 2~3 mm petiole) with 0.1% mercuric chloride. After sterilizing, shoot segment explants were washed 5~6 times in sterile distilled water, then cultured on MS basic medium. 120 shoot segments were treated each time. In 30 days, the survival rate and contaminating rate were investigated.

Branches collected in Sep. do not need water culture. Sterilizing treatment is the same as above. Shoot segment were sterilized 6 min, cultured in 4 kinds of mediums: MS, MS + 0.05 mg/L BA, MS + 0.1 mg/L BA + 0.01mg/L NAA, and MS + 0.5 mg/kg BA + 0.05mg/L NAA. Contaminating rate and survival rate were counted in 30 days.

Initial culture and transplant times

The micro-cutting explants were cultured initially on six mediums as follows:

A1: MS+0.5mg/L BA+0.05 mg/L NAA
A2: MS+1.0 mg/L BA+0.1 mg/L NAA
A3: WPM+0.5 mg/L BA+0.05 mg/L NAA
A4; WPM+1.0 mg/L BA+0.1 mg/L NAA
A5: SH+0.5 mg/L BA+0.05 mg/L NAA
A6: SH+1.0 mg/L BA+0.1 mg/L NAA

According to the results, initial culture plantlets were separated, or cut into shoot segments, and inoculated on A1, A3, and A5 mediums with hormone of low concentration. The last micro-cuttings were transplant on medium SH+0.3 mg/L BA+0.03 mg/L NAA+150mg/L LH. Twenty micro-cuttings were treated each time. After 30 d the growth status of seedlings was investigated and reproduction coefficient was counted.

Selection of subculture mediums

The micro-cuttings were transplanted onto the following subculture mediums so as to choose the optimal medium.

C1: MS+1.0 mg/L BA+0.05 mg/L NAA+10 mg/L Glu.
C2: 1/2 MS+1.0 mg/L BA+0.05 mg/L NAA+10 mg/L Glu
C3: MS
C4: 1/2MS+0.5 mg/L BA+0.05 mg/L NAA+10 mg/L Glu.
C5: WPM+0.5 mg/L BA0.5+0.05 mg/L NAA+10 mg/L Glu.
C6: B_5+0.5 mg/L BA+0.05 mg/L NAA+ 10 mg/L Glu.
C7: SH+0.5 mg/L BA+0.05 mg/L NAA+0 mg/L Glu.

All the mediums mentioned above were supplemented with 0.6% agar, and 3% sugar. The pH value was in 5.8~6.2. The culture was conducted at temperature of (23±2)℃, illumination of 12-16 h, and intensity of illumination 2000~3000 lx. Propagation coefficient was counted after culturing 30 d.

Rooting culture

The subculture seedlings were inoculated on four kinds of rooting mediums:

1/2MS+0.1 mg/L NAA+10 mg/L Glu.
1/2MS+0.2 mg/L NAA+10 mg/L Glu.
1/2MS+0.5 mg/L NAA+10 mg/L Glu.
1/2MS+1.0 mg/L NAA+ 10 mg/L Glu.

The entire mediums were supplemented with 0.6% agar, and 2% sugar. Rooting percentage was determined in 30 days.

The way of forming seedling with callus

Because a great number of callus have been produced in initial culture, the forming seedling experiment with callus was conducted. 40 pieces of callus were inoculated on each medium of MS+0.01 mg/L 2,4-D, MS+0.05 mg/L 2,4-D, MS+0.1 mg/L 2,4-D, and MS, and the investigation on forming seedlings was made in 30 days. In addition, the callus with bast from explants sprouting were cut into segments and inoculated on mediums of C1, C2, C5, and C6, at same time, 30 pieces of green dense callus from branch base and leave explants were also inoculated on the above mediums. Observation and investigation were made in 30 days.

Result and analysis

Explant induction

The survival rate of explants inducted in spring was 73.7% and 36.0% respectively for 3-min and 5-min sterilizing and the contaminative rate was 21.1% and 13.0% respectively for 3-min and 5-min sterilizing (see Table 1). As a result, 3-min sterilizing should be selected for higher survival rate of explants in spring.

Table 1. Sterilize time and contaminant rate of *R. pseudoacacia* f. *decaisneana* explants in spring

Sterilizing time (min)	Number of inoculation	Contaminative rate (%)	mortality (%)	Survival rate (%)
5 (with pitiole)	120	13.0	51	36.0
3 (without pitiole)	120	21.1	5.2	73.7

Explant culture results on 4 kinds of mediums in autumn are given in Table 2. The mean contaminative rate was 9.2% for 6-min sterilizing, lower then that of 5-min sterilizing

in spring. The mortality (including non-growth) was 42.8%, 23.3%, 17.8%, and 13.6%, respectively on four kinds of mediums (see Table 2), with a mean value of 25%. High mortality in autumn may be due to that the sterilizing time is too long or that the endogenous hormone cause explants to be in dormancy and not easy to differentiate. The forming callus occupied 49% of inoculating number in autumn, which exposed the explants is in transition period from differentiation to dormancy. Low survival rate (16.7% in average) revealed lower differentiation in autumn.

Table 2. Shoot explants culture of *R. pseudoacacia* f. *decaisneana* in autumn

Medium	Number of inoculation	Number of Contamination	Number of callus and forming plantlet	Survival rate (%)	Number of Callus	Number of died and non-growth	Mortality (%)
MS,	28	1	3	10.7	12	12	42.8
MS+BA0.05	30	3	6	20.0	14	7	23.3
MS+BA0.1+ NAA0.01,	28	5	3	10.7	15	5	17.8
MS+BA0.5+ NAA0.05	22	1	6	27.2	12	3	13.6

Initial culture and times of transplant

Initial culture results of *R. pseudoacacia* f. *decaisneana* are shown in Table 3. A5 medium was the optimal medium for initial culture, with an induction rate of forming plantlet of 75%, a propagation coefficient of 4.1 per micro-cutting in a month. Under the same basic medium and the same ratio between BA and NAA but different concentration of hormone, for instance, mediums of A1 and A2 (Table 3), the propagation coefficient was nearly same.

After twice of transplant, the propagation coefficient decreased from 4.1 of initial culture to 1.4 of second transplants culture on the SH basic medium, brown and black callus was big, and plantlet growth was poor, even died. As a result, it is suggested that transplant on the same medium cannot be over 3 times.

Table 3. Initial culture of *R. pseudoacacia* f *decaisneana* in spring

Medium		Number of inoculation	Number of survival	Number of forming plantlet	Propagation coefficient
A1	MS+0.5mg/L BA+0.05 mg/L NAA	20	15	44	2.9
A2	MS+1.0 mg/L BA+0.1 mg/L NAA	20	19	54	2.8
A3	WPM+0.5 mg/L BA+0.05 mg/L NAA	20	15	30	2.0
A4	WPM+1.0 mg/L BA+0.1 mg/L NAA	20	15	32	2.1
A5	SH+0.5 mg/L BA+0.05 mg/L NAA	20	15	61	4.1
A6	SH+1.0 mg/L BA+0.1 mg/L NAA	20	16	60	3.8

Selection of subculture mediums

The micro-cuttings were transplanted on 7 subculture mediums and the results were shown in Table 4. From Table 4, it can be seen that the medium B_5+0.5 mg/L BA+0.05mg/L NAA+10 mg/L Glu. was the best medium and had a propagation coefficient of 4.7, which was higher than propagation coefficient (4.1) on SH basic medium in spring. In order to determine the effects of medium on propagation coefficient, statistical analysis of variance was made ($F=22.81>F_{0.05}(4,46)=3.22$). It is concluded that the effects of medium on propagation coefficient was significant. Multiple comparison showed that propagation coefficient on medium C6 was larger than that on mediums of C1, C2, C3, C4, and C7 (Table 5), but there is not significant difference between on medium C6 and on medium C5. Propagation coefficient on medium C5 was significant higher than that on mediums of C3, C4, and C7, but there is not significant difference between on medium C5 and on mediums of C1, C2.

Table 4. Subculture selection of *R. pseudoacacia* f. *decaisneana*

Medium	Number of inoculation	Number of survival	Plantlet number formed	Propagation coefficient	Characteristics
C1: MS+BA1.0+NAA0.05+Glu.10	8	21	55	2.6	olivine seedling lodging, light-brown big callus
C2: 1/2 MS+BA1.0+NAA0.05+ Glu.10	8	21	48	2.24	olivine seedling lodging, light-brown big callus
C3: MS	7	23	33	1.43	olivine seedling lodging, brown and big callus
C4: 1/2MS+BA0.5+NAA0.05+Glu.10	8	21	35	1.67	seedling grow bad and lodging, big callus
C5: WPM+BA0.5+NAA0.05+Glu.10	8	22	75	3.4	stronger olivine seedling , small callus
C6: B_5+BA0.5+NAA0.05+Glu.10	7	18	85	4.7	strong green seedling, small callus
C7: SH+BA0.5+NAA0.05+Glu.10	7	32	32	1.28	infirm yellow seedling ,even die

Plantlet forming from callus

Light brown and scatter callus cannot form plantlet on every experiment medium. This exposed that light brown and scatter callus had lost capacity of differentiation. Table 6 showed that green dense callus could form plantlet, with a plantlet forming rate of 33% on mediums of C1 and C6. Bast with callus cultured on the C6 medium had a plantlet-forming rate of 22%. Leaves also formed dense callus on medium C6. So B_5+BA0.5+NAA0.05+Glu.10 medium is the best basic medium for *R. pseudoacacia* f. *decaisneana*. The results in Table 6 also showed that the callus from different parts of plantlet have effect on induction of callus and frequency of callus differentiation.

Table 5. Multiple comparison of propagation coefficient of *R. pseudoacacia* f. *decaisneana*

Medium	Index	X_i—C7	X_i—C3	X_i—C4	X_i—C2	X_i—C1	X_i—C5
C6	4.7	3.46**	3.26**	3.01**	2.46**	2.1**	1.26
C5	3.44	2.2**	2.0**	1.75**	1.20	0.84	
C1	2.6	1.36	1.16	0.91	0.36		
C2	2.24	1	0.8	0.55			
C4	1.69	0.45	0.25				
C3	1.44	0.2					
C7	1.24						

Table 6. The other forming seedling ways of *R. pseudoacacia* f. *decaisneana*

Medium	Plantlet forming rate of dense callus (%)	Forming plant rate of bast callus (%)	Forming callus by leaves
C1: MS+BA1.0+NAA0.05+Glu.10	33	3.5	0
C2:1/2MS+BA1.0+NAA0.05+Glu.10	0	12.8	0
C7: SH+BA0.5+NAA0.05+Glu.10	0	0	0
C6: B_5+BA0.5+NAA0.05+Glu.10	33	22	Formed dense and close callus

Rooting culture

Rooting results of *R. pseudoacacia* f. *decaisneana* on 4 kinds of mediums are shown in Table 7: The medium of 1/2MS+0.5 mg/L NAA + 10 mg/L Glu. was the best rooting medium, with a rooting rate of 84.4%, characterized by long main root, excessive side root, and small callus.

Table 7 Rooting results of *R. pseudoacacia* f. *decaisneana* on different mediums

Medium	Inoculating number	Rootage number	Rooting rate (%)	Average root length (cm)	Characteristics
1/2MS+NAA0.1+Glu.10	30	18	60.0	2.9	Bigger callus, short main root, less side root.
1/2MS+NAA0.2+Glu.10	34	28	82.5	3.3	Small callus, long main root, more side root.
1/2MS+NAA0.5+Glu.10	32	27	84.4	3.2	Bigger callus, long main root, more side root.
1/2MS+NAA1.0+ Glu.10	31	23	74.2	3.6	Bigger callus, long main root, less side root.

Conclusion and discussion

The culture of shoot segment explant *R. pseudoacacia f. decaisneana* in spring is better than in autumn. Although it had a higher contaminated rate, abduction survival rate was high. Kamlesh (1997) and Civinova (1990) reported that explants inducing culture was effective in spring.

On the medium of SH+0.5 mg/L BA+0.05 mg/L NAA, the reproducing rate of *R. pseudoacacia f. decaisneana* was up to 4.1. With the culture going on, it is seriously degenerate. The reproducing rate was only 1.4 on SH basic medium after two times of transplan, callus was big, and seedling changed to brown and distortion, even dies, is the symbol of aging (Xu 1991 and 1992), but the reason of becoming brown is very complex (Luo 1999). Poor Growth on subculture medium SH+0.5 mg/L BA+ 0.05 mg/L NAA+10 mg/L Glu. indicated that transplant on the same medium cannot be over 3 times. Selection of subculture medium showed the medium B_5+0.5 mg/L BA+0.05 mg/L NAA0.05+10 mg/L Glu. was the best medium and had the highest propagation coefficient (4.7).

The callus which is light-brown, blanch or sparse cannot form seedling. Green and denseness callus can form seedling on the medium of B_5+0.5 mg/L BA+0.05 mg/L NAA+ 10 mg/L Glu. and the rate of forming seedling is very high. Leaves can form green and denseness callus (Li, 1992).

The medium of 1/2MS+0.5 mg/L NAA+ 10 mg/L Glu. was the best rooting medium, with a rooting rate of 84.4%, and characterized by long main root, excessive side root, and small callus

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